See also: **II / Chromatography: Thin-Layer (Planar):** Densitometry and Image Analysis; Layers; Modes of Development: Conventional; Modes of Development: Force Flow, Overpressured Layer, Chromatography and Centrifugal; Spray Reagents. **III / Amines: Gas Chromatography. Impregnation Techniques: Thin-Layer (Planar) Chromatography. Pharmaceuticals: Basic Drugs: Liquid Chromatography**.

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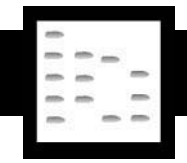
BILE ACIDS



Gas Chromatography

A. K. Batta and G. Salen,
UMD-NJ Medical School, Newark, NJ and VA Medical Center, East Orange, NJ, USA

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Introduction

Bile acids, a group of steroidal acids with a carboxyl group in the side chain, are the major end products of cholesterol catabolism, formed in the liver, conjugated with amino acids, glycine and taurine, and secreted into the bile. In most animal species, bile acids contain 24 carbons, with the terminal side chain carbon in the form of a carboxyl group; however, certain reptiles have 27-carbon bile acids as the major biliary bile acids. Primary bile acids are formed via the 5β-saturation of cholesterol double bond by hepatic enzymes, epimerization of 3β-hydroxyl group to α-configuration and further insertion of 7α- and/or 12α-hydroxyl group, shortening of the side chain by three carbons and oxidation of the terminal carbon to a carboxyl group. Structures of some of the bile acids found in animal species are shown in **Figure 1**. Bile acids facilitate the absorption of dietary lipids, including fat-soluble vitamins and cholesterol, via their detergent action. The detergent properties of bile acids result from their unique structure with a nonpolar steroid skeleton and a polar carboxyl group and α-oriented hydroxyl groups, further increased by hepatic conjugation with glycine and taurine (Figure 1). Bile acid conjugates form micelles with phospholipids that solubilize cholesterol in the bile. The primary bile acids, cholic acid and chenodeoxycholic acid, are effectively reabsorbed from ileum during their enterohepatic circulation, but approximately 5% that escape reabsorption seep into the colon, and are subjected to modification to secondary bile acids by intestinal bacteria. These modified bile acids, in particular the 7α-dehydroxylated bile acids, lithocholic acid and deoxycholic acid, are the major faecal bile acids and are also significantly absorbed from the colon and circulate in the enterohepatic circulation, with deoxycholic acid as one of the major plasma and biliary bile acids in humans. Whereas only small amounts of bile acids are excreted into the urine, approximately 500 mg per day is excreted in faeces and forms a major catabolic pathway for the elimination of body cholesterol.

In hepatobiliary and intestinal diseases, the hepatic synthesis and clearance of bile acids and their intestinal absorption are abnormal, which disturbs both cholesterol synthesis and its metabolism, causing increased plasma, urinary and faecal concentrations of bile acids. This results in accumulation of precursors of cholesterol or bile acids and clinical malformations ensue. Early diagnosis of such conditions is often possible from bile acid analysis in bile, serum, urine and faeces. On the other hand, bile acids have therapeutic applications in conditions of abnormal cholesterol biosynthesis and metabolism, and chenodeoxycholic acid, and its 7β-hydroxy epimer, ursodeoxycholic acid, are used for medical treatment of gallstones while ursodeoxycholic acid is also being

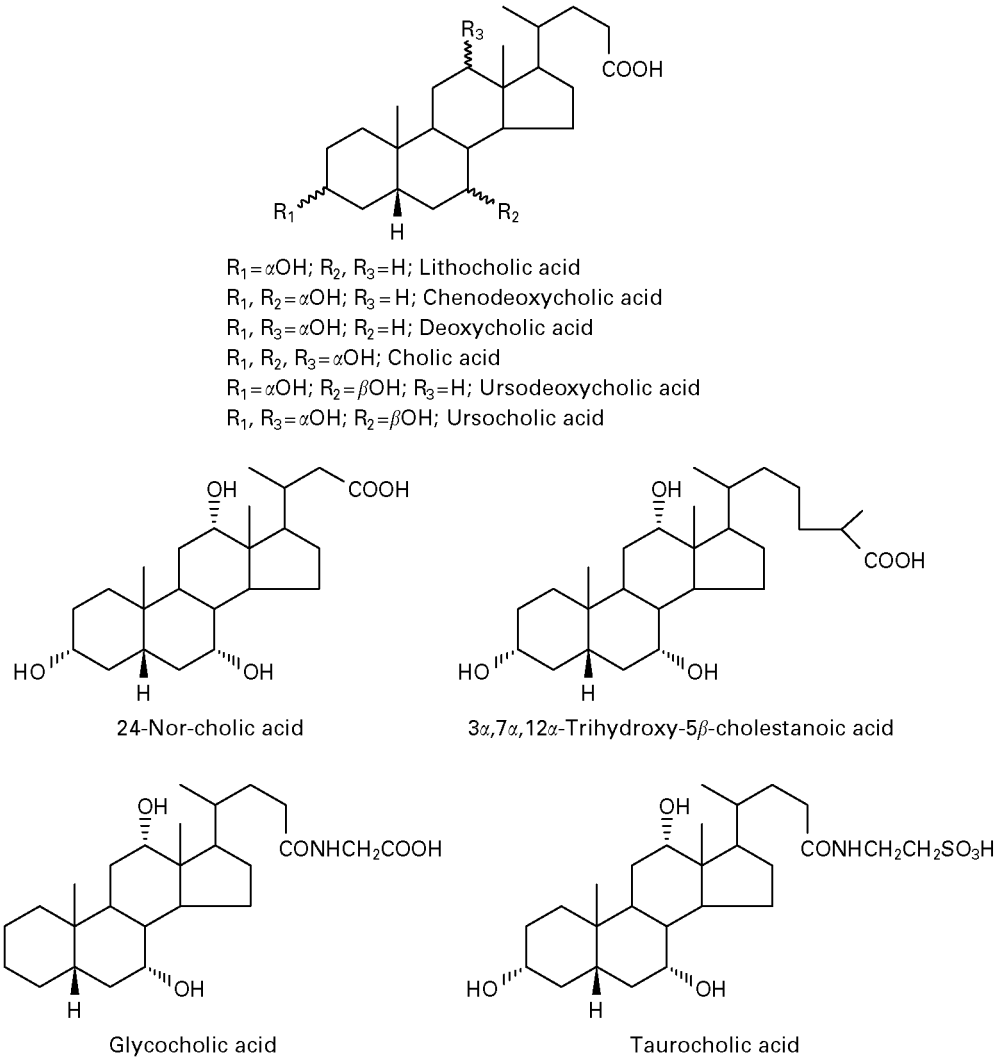

Figure 1 Structures of bile acids and their derivatives.

used in clinical trials for a variety of hepatobiliary diseases, including primary biliary cirrhosis, primary sclerosing cholangitis and hepatitis C, and is suggested to suppress colon polyp formation in experimental animals. Thus, bile acid analysis is an important diagnostic tool for diseases of the liver and intestine, and for monitoring bile acid therapy in such diseases. Of the several methods employed for bile acid analysis in biological fluids, gas chromatography (GC) has proven to be the most versatile and is sensitive enough to quantitate nanomole to picomole amounts present in biological specimens.

Choice of Column

For more than a decade, since the first application of GC for bile acid analysis by Vanden Heuvel *et al.* in 1960, GC was performed using metal or glass-packed columns. Introduction of capillary or open tubular columns was a major landmark in the development of GC. The separation of serum bile acids using capillary columns was first demonstrated by Laatikainen *et al.* in 1975 (**Table 1**). Coupled with the fact that the retention times are highly reproducible, capillary columns have fast become columns of choice for most chromatographic applications for bile acids. The usual capillary column is 25–50 m long with an internal diameter of 0.22–0.25 mm. The inner wall is coated with the liquid phase, which may vary from nonpolar (e.g. methyl silicones, such as OV-1, CP-Sil-5 CB and SE-30) to more polar (like phenylmethyl- and cyanopropylsilicone, e.g. CP-Sil-19 CB) depending on the need. Usually peaks are very sharp; however, the dead volume must be very small and

Table 1 Important advances in gas chromatography of bile acids

1960–61	Vanden Heuvel *et al.*	First application of gas chromatography for bile acid analysis. Bile acid methyl ester-trifluoroacetates were analysed on packed columns
1965	Grundy *et al.*	Comprehensive method for faecal bile acid analysis using packed columns. Bile acids were isolated free from faecal fatty acids, sterols and other contaminants before chromatography. Method often used as a standard to which other methods are compared
1975	Laatikainen *et al.*	Introduction of capillary columns for serum bile acid analysis
1987	Child *et al.*	Simultaneous quantitation of faecal bile acids, sterols and fatty acids
1998	Batta *et al.*	Bile acid analysis was greatly simplified. Bile acids were derivatized and quantitated directly in the stool sample and the multiple steps of removal of sterols and fatty acids were avoided

only microgram or nanogram quantities of compounds can be injected in order to get appropriate peak shapes.

New instrumentation and use of splitless injectors have greatly reduced the dead volume while increasing the sensitivity, so that capillary columns are ideal for chromatography of trace compounds in biological fluids. Quantitation of bile acids in a sample is carried out by comparison of peak areas with those of known amounts of reference compounds in a range where a linear detector response is obtained. Usually a known amount of an internal and/or external standard is added to the sample to facilitate quantitation. Often a mass spectrometer is used as a detector for GC, and gas chromatography–mass spectrometry (GC-MS) has the added advantage that the mass fragmentation can give insight to the structure of the compound. The sensitivity of GC-MS is several-fold increased by using selected-ion monitoring, when picomole quantities of bile acids can be detected. Selection of the right internal standard is important for quantitation of bile acids using the mass spectrometer in the selected-ion mode. Often isotope-labelled bile acids (polydeuterated) are used, the advantage being that such compounds mix with the bile acid to be quantitated and are similarly extracted from the biological source. Otherwise, a bile acid with different GC retention time is employed, and peak heights are calibrated using known amounts of the compounds to be quantitated. The mass ion selected for quantitation is usually a high mass ion and with significant abundance.

Extraction of Bile Acids

Bile acids are present from 1–2 $\mu g\,mL^{-1}$ in plasma and urine to significant amounts in the intestinal content and as much as 80 $mg\,mL^{-1}$ in the gall bladder bile. They are present in unconjugated form as well as conjugated with glycine and taurine or as sulfate/glucuronides, often in association with proteins, sterols and their esters, free or esterified fatty acids, bile pigments and water-soluble small molecules, which may interfere in their analysis. It is often necessary to isolate and at least partially purify bile acids before preparation for GC. Plasma bile acids mainly exist as glycine and taurine conjugates and unconjugated bile acids are present in only small proportions. Plasma is usually passed through Sep-pak, a reversed-phase C_{18} cartridge, when proteins and most of the cholesterol are removed and bile acids and their conjugates are eluted with methanol. Other methods of concentrating bile acids include lipophilic anion exchange gel, diethylaminohydroxypropyl Sephadex LH-20 and, more recently, size exclusion chromatography using Sephadex G-75 gel. Some of these ion exchange resins are also employed for separation of various conjugated bile acids so that conjugation pattern is determined in the sample. The bile acid fraction obtained by any of these methods is treated with strong alkali (4 $mol\,L^{-1}$ sodium hydroxide, 115°C at 15 psi pressure) to hydrolyse the conjugates; neutral sterols are extracted out with solvents and the free bile acids are then extracted with ether or ethyl acetate after acidification. Under these conditions, the glucuronides and sulfate esters are also hydrolysed, and a pre-step of solvolysis may not be necessary. As an alternative to alkaline hydrolysis, bile acid conjugates may be hydrolysed with the enzyme, cholylglycine hydrolase, while β-glucuronidase plus sulfatase is used to hydrolyse glucuronides and sulfates. In order to correct for losses during extraction, an internal standard is added before the hydrolysis step, while an external standard (usually 5α-cholestane) corrects for detector responses of the various compounds. The appropriate internal standard is a bile acid that is not present in the bile acid mixture to be quantitated. Nor-deoxycholic acid and norcholic acid are usually used as internal standards, but other compounds like hyodeoxycholic acid and 7α, 12α-dihydroxy-5β-cholanoic acid have also been employed.

Biliary bile acids are present predominantly as the glycine and taurine conjugates. Since they are present in high concentrations in the bile, only a few microlitres of bile are usually required for quantitation by

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GC. Also, since bile contains only 1–2% of cholesterol, it is much easier to obtain pure bile acids free from cholesterol. Urinary bile acids are also present mainly in conjugated form, and only a few milligrams per day are excreted. However, in hepatobiliary diseases like primary biliary cirrhosis, urine may become a major pathway for bile acid excretion and sulfate conjugation of bile acids is increased. Again, bile acids need to be deconjugated before GC analysis and methods similar to those for plasma can be used to obtain urinary bile acid composition.

Faecal bile acids are mainly found in unconjugated form and the bile acid pattern is highly complex, due to bacterial deconjugation and extensive metabolism during intestinal transit. Most abundant of these secondary bile acids are deoxycholic acid and lithocholic acid, which are reabsorbed from the colon and further modified by hepatic enzymes and circulate in enterohepatic circulation. Whereas scores of metabolites of bile acids are formed in the colon, bile acids in the jejunum remain predominantly conjugated due to the absence of bacteria in the small intestine, and they therefore mirror biliary bile acids. A major difficulty in quantitative analysis of faecal bile acids is their strong binding with the bacterial debris in the stool, and quantitative extraction is difficult. Furthermore, in addition to bile acids, stool contains neutral sterols, including cholesterol and its bacterial metabolites and plant sterols and their bacterial metabolites, and also fatty acids. Several methods have been reported for bile acid extraction from faeces, and most are quite complex. Thus, one method involves continuous Soxhlet extraction of aliquots of homogenized stool with chloroform–methanol; the extracted bile acids are subjected to methyl ester formation followed by preparative thin-layer chromatography. Grundy *et al*. extracted neutral sterols from homogenized stool, deconjugated any bile acid conjugates with alkali and finally extracted bile acids with chloroform–methanol after acidification of the solution. After evaporation of solvents, the residue was chromatographed over Florisil and the purified bile acid fraction was subjected to methyl ester formation and then to preparative TLC to separate fatty acids. Bands due to bile acids were isolated and used for GC. In other methods, stools have been extracted with ammoniacal alcohol, methanol–hydrochloric acid, acetic acid–toluene, and bile acids extracted after removal of neutral sterols.

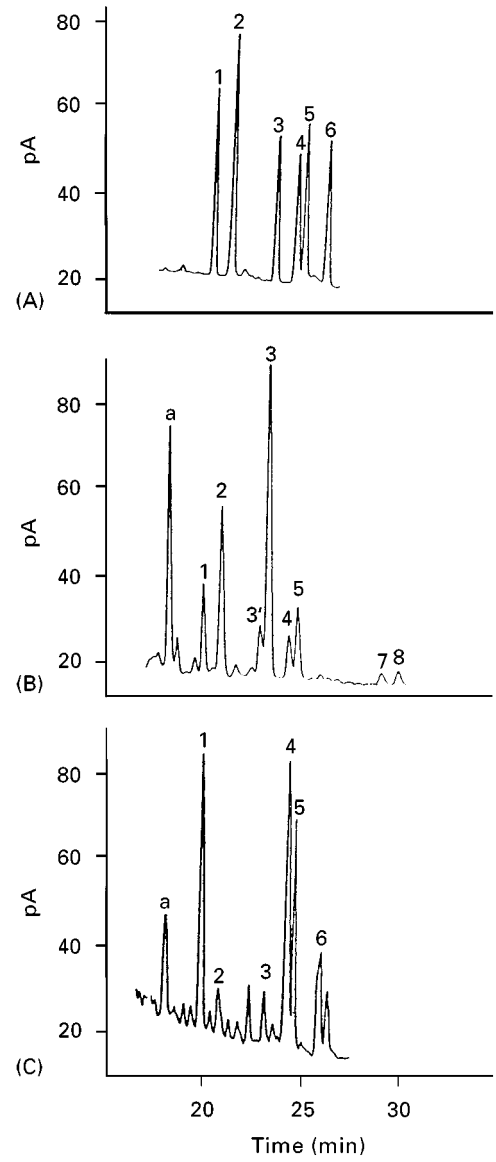

Figure 2 Gas chromatography of bile acids. Chromatographic conditions are described in Table 2; CP-Sil-5 CB capillary column was employed. (A) *n*-Butyl ester-trimethylsilyl ether derivatives of standard bile acids. Peak identification, derivatives of: 1, nor-cholic acid; 2, lithocholic acid; 3, deoxycholic acid; 4, chenodeoxycholic acid; 5, cholic acid; 6, ursodeoxycholic acid. (B) Faecal bile acids in a healthy volunteer. Dried stool (10 mg) containing 20 μg nor-cholic acid as internal standard was directly subjected to *n*-butyl ester followed by trimethylsilyl ether formation, dissolved in 200 μL hexane and 1 μL was injected into the GC column. Peak identification: 1–6, same as in (A); a, sitosterol, 3′, isodeoxycholic acid; 7, 3-keto, 12α-hydroxy-5β-cholanoic acid; 8, 12-ketolithocholic acid (C) Plasma bile acids in a patient with lipid storage disease, sitosterolemia. Plasma (1 mL) containing 10 μg nor-cholic acid as internal standard was used. Bile acids were enzymatically deconjugated and bile acids obtained by passing through Sep-pak. Bile acids were derivatized as *n*-butyl ester-trimethylsilyl ethers, dissolved in 100 μL hexane and 5 μL was injected into the GC column. Peak identification: 1–6, same as in (A); a, sitosterol.

Derivatization of Bile Acids

Derivatization of Carboxyl Group

The carboxyl group in bile acids is most often converted into the methyl ester. Treatment with

Table 2 GC retention indices of bile acids as their methyl ester-trimethylsilyl ether derivatives[a]

Bile acid methyl ester-trimethylsilyl ether	Retention index[b] CP-Sil-5 CB	CP-Sil-19 CB
Lithocholic acid	3157	3339
Deoxycholic acid	3221	3373
Chenodeoxycholic acid	3244	3397
Cholic acid	3261	3381
Ursodeoxycholic acid	3279	3439
Hyodeoxycholic acid	3256	3422
Hyocholic acid	3340	3445
β-Muricholic acid	3310	3468
Nor-cholic acid	3140	
Nor-deoxycholic acid	3106	
Homocholic acid	3346	
3α,7α,12α-Trihydroxy-5β-cholestanoic acid	3468	

[a]A Hewlett-Packard model 6890 gas chromatograph equipped with a flame ionization detector and an injector with a split/splitless device for capillary columns was used. A chemically bonded fused silica CP-Sil-5 CB or CP-Sil-19 CB capillary column (25 m $\times$ 0.22 mm i.d.) was employed and helium was used as the carrier gas. The column temperature was kept at 100°C for 2 min, then increased at a rate of 35°C min^{-1} to a final temperature of 278°C.
[b]Retention indices (Kovats values) were determined by previous injection of an alkane mixture under identical GC conditions.

diazomethane in ether converts bile acids instantly into their methyl esters. However, small amounts of methyl ethers are formed as artefacts, so alternative methods are often employed. Methyl esters are quantitatively formed with methanol–sulfuric acid or anhydrous methanolic hydrochloric acid. Alternatively, 2,2-dimethoxypropane-hydrochloric acid may be used for derivatization. In addition to methyl esters, ethyl, *n*-propyl, isobutyl and *n*-butyl esters have all been employed, the advantage being that the retention times of the bile acid derivatives are increased and, sometimes, resolution is improved. In particular, much better separations from neutral sterols can be obtained and, as shown in **Figure 2**, chemical removal of neutral sterols may be completely unnecessary with the use of butyl esters.

Derivatization of Hydroxyl Groups

The derivative of choice for the hydroxyl group is the trimethylsilyl ether and a variety of silylating reagents are commercially available for quantitative derivatization of even hindered and tertiary hydroxyl groups. Thus, *N*,*O*-bis(trimethylsilyl)acetamide, in combination with trimethylchlorosilane, can convert relatively unhindered hydroxyl groups into their trimethylsilyl ether derivatives and, when mixed with trimethyliodosilane, the reagent is highly potent and can silylate hydroxyl groups in all positions. Bis(trimethylsilyl)trifluoroacetamide is equally reactive as *N*,*O*-bis(trimethylsilyl)acetamide but the reagent and the by-products are more volatile. The older silylating reagent, a mixture of 1,1,1,3,3,3-hexamethyldisilazane, trimethylchlorosilane and pyridine (3 : 1 : 9) is still commonly used for derivatization. Retention volumes of several common bile acid methyl ester-silyl ether derivatives on capillary

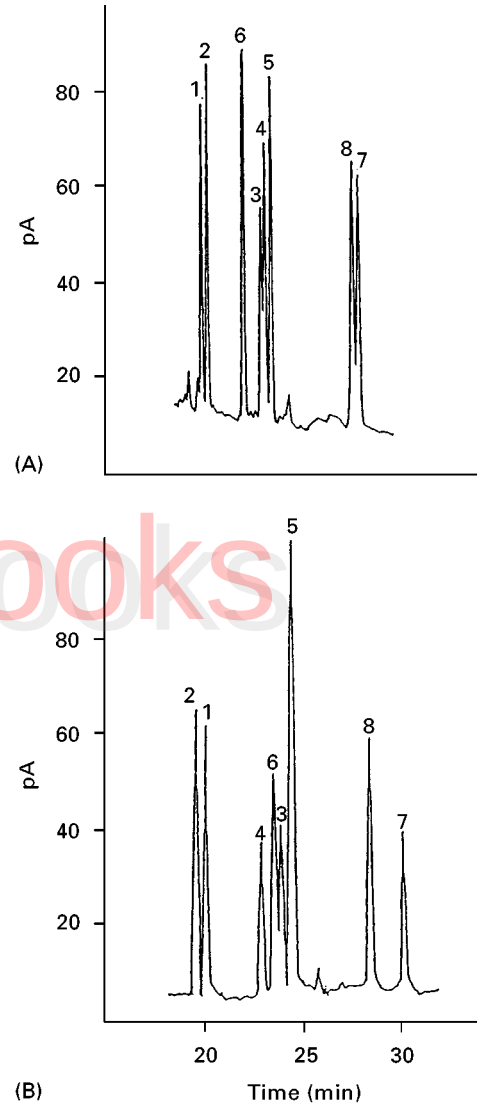

Figure 3 Gas chromatography of 6-hydroxylated bile acids. Chromatographic conditions are described in Table 2. (A) CP-Sil-5 CB capillary column; (B) CP-Sil-19 CB capillary column. Peak identification, derivatives of: 1, 3α,6β,7α-trihydroxy-5β-cholanoic acid; 2, 3α,6β,7α,12α-tetrahydroxy-5β-cholanoic acid; 3, 3α,6α,7α-trihydroxy-5β-cholanoic acid; 4, 3α,6α,7α,12α-tetrahydroxy-5β-cholanoic acid; 5, 3α,6β,7β-trihydroxy-5β-cholanoic acid; 6, 3α,6β,7β,12α-tetrahydroxy-5β-cholanoic acid; 7, 3α,6α,7β-trihydroxy-5β-cholanoic acid; 8, 3α,6α,7β,12α-tetrahydroxy-5β-cholanoic acid.

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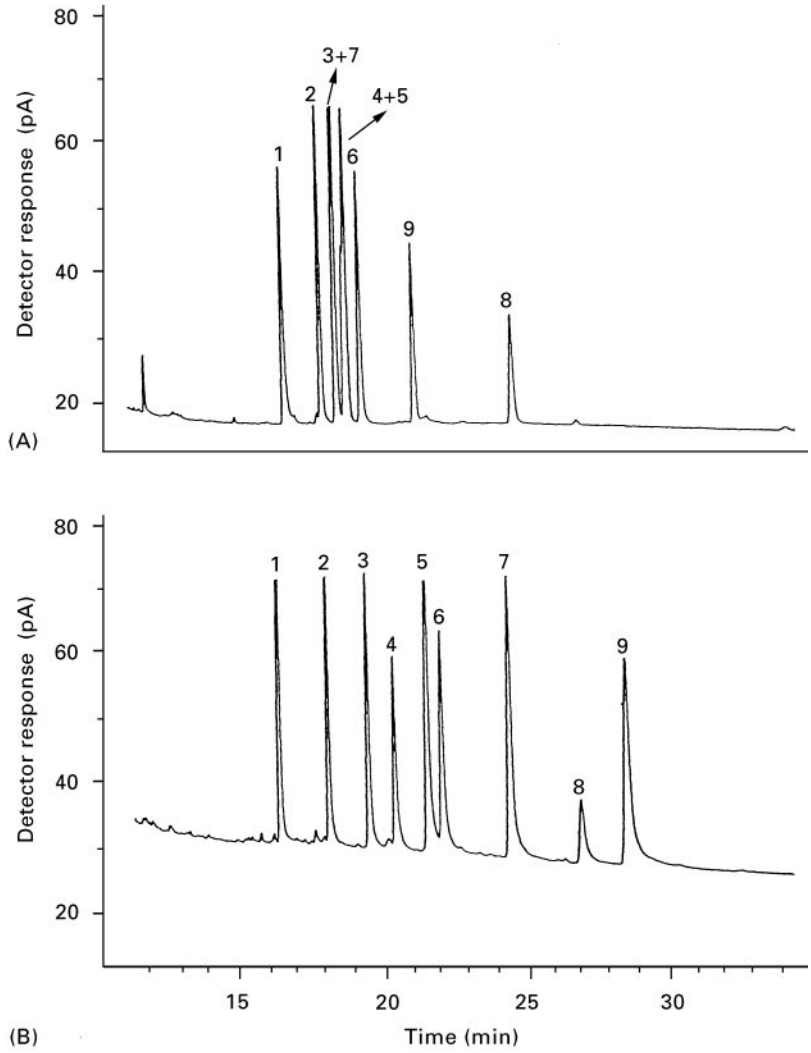

Figure 4 Gas chromatography of acetate-methyl esters and trimethylsilyl ether-methyl esters of bile acids. Chromatographic conditions are described in Table 2; CP-Sil-5 CB capillary column was employed. (A) Trimethylsilyl ether-methyl esters of bile acids. (B) Acetate-methyl esters of bile acids. Peak identification: 1, lithocholic acid; 2, deoxycholic acid; 3, chenodeoxycholic acid; 4, cholic acid; 5, 3α,6α-dihydroxy-5β-cholanoic acid; 6, ursodeoxycholic acid; 7, 3α,6β,7α-trihydroxy-5β-cholanoic acid; 8, 3α,6α,7β-trihydroxy-5β-cholanoic acid; 9, 3α,6β,7β-trihydroxy-5β-cholanoic acid.

columns of low and medium polarity are given in **Table 2**, while the chromatographic resolution of these derivatives of a number of bile acids with additional hydroxyl group at C_6 (present in several animal species and in patients with hepatobiliary diseases) is shown in **Figure 3**. Although trimethylsilyl ethers have gained general applicability in bile acid analysis, other silylating groups sometimes have advantages. Thus, dimethylethylsilyl and dimethylpropylsilyl ether derivatives have longer retention times than the corresponding trimethylsilyl ethers, while *t*-butyldimethylsilyl ether derivatives are very stable, and also show significantly longer retention times, so that often better resolutions can be obtained. Hydroxyl groups can also be protected as acyl derivatives and formate, acetate and trifluoroacetate derivatives have all been employed for GC. These derivatives are often more stable than the silyl derivative and sometimes show better resolution, as seen in **Figure 4**. A one-step derivatization of the hydroxyl and carboxyl groups with heptafluorobutyric anhydride, also resulting in simultaneous hydrolysis of the glycine and taurine conjugates of the bile acids, has been reported.

Derivatization of Oxo Groups

Oxo bile acids are formed by bacterial modification of bile acids during their intestinal transit and are therefore found in the intestinal content. Quantitation of

oxo bile acids is beset with problems. Thus, oxo bile acids, in particular, 3-oxo bile acids, are vulnerable to rigorous alkaline hydrolysis conditions and therefore the much milder enzymatic hydrolysis of conjugates is preferred when oxo bile acids are suspected. Oxo bile acid methyl esters can be chromatographed without further derivatization (the hydroxyl groups in the partially oxidized compounds must be derivatized); however, sometimes artefacts are produced and it may be better to protect the oxo groups as the *O*-methyloxime or the dimethylhydrazone derivatives.

Summary

Detection and quantitation of bile acids are important in health and disease. Bile acid synthetic defects may arise from both defective cholesterol synthesis and metabolism; bile acid therapy may correct some of these abnormalities. GC has proved to be of vital importance in determination of bile acids in biological fluids. However, a complete GC analysis generally takes a long time. By appropriate column and derivatization selection, analysis time can be significantly reduced, but sample preparation for GC analysis is usually time-consuming. Combination of GC and mass spectrometry greatly aids in the characterization of unknown bile acids. In attempts to get additional information, Child *et al*. have developed a method to quantitate sterols and fatty acids together with bile acids in stool samples. Advantage is taken of the greatly increased retention times of the *n*-butyl ester-acetates of bile acids, so that sterol acetates are eluted earlier than all bile acids present and a group separation of faecal fatty acids, sterols and bile acids is achieved. In a further advancement of the method, Batta *et al*. have prepared *n*-butyl esters of bile acids directly in faecal samples, followed by trimethylsilyl ether formation. They showed that faecal bile acids could be quantitatively derivatized and eluted from the faeces and bile acids could be quantitated in the presence of other faecal components. The method is also applicable to plasma bile acid measurement. Figure 2 shows plasma and faecal bile acids in a healthy volunteer determined as the *n*-butyl ester-trimethylsilyl ether derivatives. Since the extra steps of extraction of bile acids and their purification are eliminated, the method can easily be adapted for routine screening of samples for bile acid analysis.

See also: **II/Chromatography: Gas:** Delectors: Mass Spectometry; Derivatization; **Solid Phase Extraction. III/Acids:** Gas Chromatography; Liquid Chromatography; Thin-layer (Planar) Chromatography. **Extraction:** Supercritical Fluid Extraction.

Further Reading

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Liquid Chromatography

K. Saar, Aventis Research & Technology, Frankfurt, Germany
S. Müllner, Enzyme Technology, Düsseldorf, Germany

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Introduction

Bile acids, the major components of the bile fluid, play a significant role in lipid metabolism. After formation in the liver and storage in the gall bladder, the bile is secreted in the duodenum. Due to their chemical structure, all bile acids tend to form micelles and serve as natural detergents. In addition, bile acids are co-factors of pancreatic lipases and are pivotal for the digestion and absorption of fats and fat-soluble vitamins.

The primary bile acids cholic acid and chenodeoxycholic acid are synthesized in the liver and during their enterohepatic circulation various modifications occur. Conjugation with the amino acids taurine or glycine as well as deconjugation, sulfation, glucuronidation and modification by intestinal